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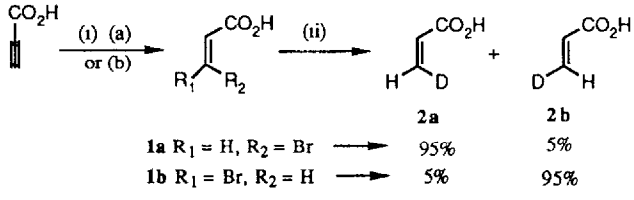
THE SYNTHESIS OF (6*R*)-[6-^{2}H]- AND (6*S*)-[6-^{2}H]5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE

Shankar Balasubramanian and Chris Abell.*
University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, England.

Summary : *(6R)-[6-^{2}H]- and (6S)-[6-^{2}H]5-enolpyruvylshikimate-3-phosphate have been synthesised for the kinetic analysis of the chorismate synthase reaction. The synthesis uses purified shikimate kinase and EPSP synthase for enantiospecific biotransformations and also clarifies the stereochemical course of the Diels Alder reaction in the synthesis of shikimic acid.*

5-Enolpyruvylshikimate-3-phosphate (**10a**, EPSP) is the substrate for the enzyme chorismate synthase (EC 4.6 1.4), the final enzyme on the common shikimate pathway.[1] Chorismate synthase catalyses the *trans* 1,4-elimination of phosphate from EPSP to produce chorismate.[2,3] The mechanism of this reaction has been the source of much speculation but still remains poorly understood.[3,4,5,6] As part of a detailed kinetic study of the chorismate synthase mechanism we required (6*R*)-[6-^{2}H]EPSP **10c**. The observation of a primary kinetic isotope effect in the chorismate synthase reaction would reveal the kinetic significance of the carbon-hydrogen cleavage step in the enzyme reaction mechanism, and be a first step towards elucidating the mechanism.

A synthesis of unlabelled EPSP has been previously reported.[7] In this paper we report the synthesis of (6*R*)-[6-^{2}H]EPSP and (6*S*)-[6-^{2}H]EPSP. The strategy uses a Diels Alder based synthesis of shikimic acid[8,9] followed by enzymic conversion to EPSP. The isotopic label is introduced from the *Z* and *E* isomers of [3-^{2}H]acrylic acid, which were synthesised from the corresponding bromoacrylic acids[10] (Scheme I).

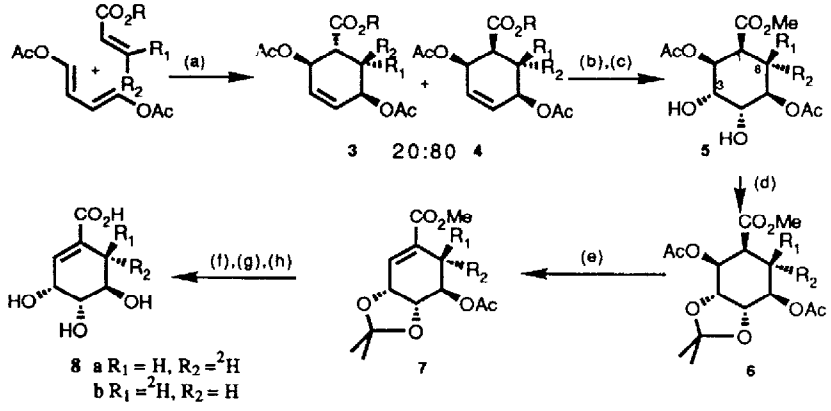


Scheme I (i) (a) 8% aq HBr, 55°C, 60%; (b) 48% aq. HBr, 140°C, 55%; (ii) 2.5 equivalents of 2 5% sodium amalgam in D_2O

The (*Z*)- and (*E*)-3-bromoacrylic acids **1a** and **1b** were synthesised by the methods of Bey and Vevert[11] and of Crout and Corkill[10] respectively. Reduction by sodium amalgam in D_2O[12] gave the corresponding [3-^{2}H]acrylates **2a** and **2b** which contained greater than 99% atomic deuterium with 95% stereoselectivity (by ^{1}H NMR spectroscopy). This preparation of deuteriated acrylates was preferred to the method published by Hill and Newkome,[13] using the Diels Alder adduct of anthracene and propiolic acid which is experimentally more difficult and did not give as high a level of stereospecific deuterium incorporation

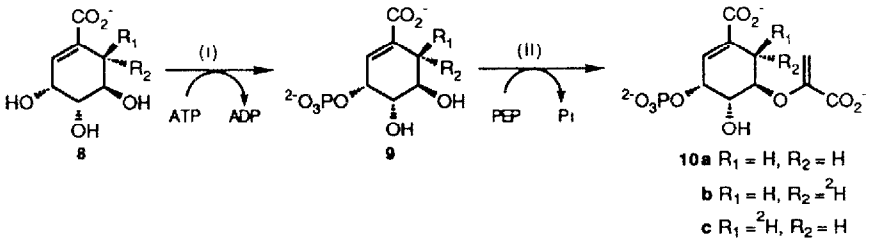
The deuteriated acrylates **2a** and **2b** were converted to the corresponding shikimates **8a** and **8b** following the route of McCrindle *et al.* [8] and Smissman *et al* [9] (Scheme II). McCrindle *et al* carried out the Diels Alder reaction of acrylic acid and (*E,E*)-1,4-diacetoxybutadiene, and the adduct formed was assigned to have *endo* stereochemistry **4** (R=H). The analogous reaction carried out by Smissman *et al.* using methyl acrylate gave what was assigned to be the *exo* adduct **3** (R=Me). This difference in stereochemical assignment is not of great importance in the synthesis of unlabelled shikimic acid but is critical in our synthesis of labelled shikimate to preserve the stereochemical integrity of the deuterium at C-6. For this reason both Diels Alder reactions were investigated under the original published conditions[8,9] and both were found to result in the formation of an approximately 80:20 mixture of distereomers, as determined by ^1H and ^{13}C NMR spectroscopy. The coupling constants in the ^1H NMR spectra of the intermediate **5** show the ester group to be *cis* to both acetoxy groups in the major isomer resulting from both Diels Alder reactions.[14] This is consistent with data published by McCrindle *et al.*[15] Further evidence was the relative ease of base catalysed elimination of acetic acid from the protected diol **6** by DBU (6 equivalents) in ether under reflux to give the protected shikimate **7** in good yield (85%). This contrasts with the pyrolytic conditions necessary to effect what was believed to be a *cis* elimination process from a derivative of the *exo* adduct.[9]

The required *endo* diastereomer **4** (R=H) was purified easily from the mixture by two recrystallisations from toluene/petroleum ether (40-60°) in an overall yield of 40%. This was then converted to shikimic acid (**8**) in 6 steps (as shown in Scheme II). The synthesis proceeds through the same intermediates as previous syntheses[8,9] but uses different reagents and proceeds in an improved overall yield of 20%. The extent of deuterium labelling in the acrylate **2a** or **2b** was retained in the isolated shikimic acid in the stereochemistry corresponding to the *endo* intermediate **4**.[16]



Scheme II (a) 90°C, **4** in 40% yield after 2 recrystallisations from toluene/petroleum ether (40-60°), (b) CH_2N_2, Et_2O, 100%; (c) aq.OsO_4(1%), $NaClO_3$ (1.5 equiv.), 65%; (d) 2,2-dimethoxypropane, p-toluene-sulphonic acid, 80%; (e) DBU (6 equiv), Et_2O reflux, 85%; (f) aq. AcOH 60%, 70°C, 60%; (g) K_2CO_3, MeOH/H_20; (h) Dowex (H^+) 80%.

The enzymes shikimate kinase and EPSP synthase catalyse the conversion of (-)-shikimate to EPSP, via shikimate-3-phosphate. In our preparation of deuteriated EPSP, shikimate kinase and EPSP synthase were each purified from an overexpressing strain of *E. coli* [17,18] and used to transform shikimate (**8**) to shikimate-3-phosphate (**9**) then EPSP (**10**) (Scheme III). The transformations were performed sequentially in an NMR tube under observation by ^1H NMR spectroscopy (Figure 1)



Scheme III (i) 1.8 U shikimate kinase, 24h. (ii) 0.08 U EPSP synthase 8h. Both transformations were carried out sequentially on 100mM racemic shikimic acid in 1ml D_2O, pD7.1 (Tris-DCl, 50mM) at 25°C containing 50mM $MgCl_2$, 50mM ATP and 50mM PEP.

The reaction conditions favoured the EPSP synthase reaction which functioned close to its V_{max}.[18] Under these conditions shikimate kinase functioned below one tenth of its optimal activity[17] hence relatively more of this enzyme was required for the transformation Both reactions were easily monitored by a downfield shift in the C-2 proton resonance on addition of each enzyme. A comparison of the integrals of the EPSP signal and the residual (+)-shikimate signal, on completion of both reactions, suggests that the overall conversion was enantiospecific and virtually quantitative.

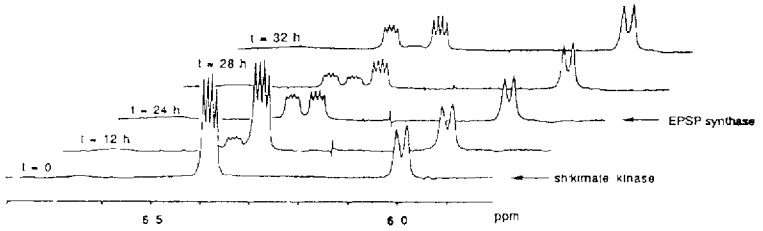


Figure 1 A timecourse of ^1H NMR spectra showing the conversion of racemic shikimate (**8**) to homochiral shikimate phosphate (**9**) and on to EPSP (**10**). (+)Shikimate does not react. Shikimate kinase is added at t=0 and EPSP synthase at t=24 h. The C-2 proton resonance occurs progressively further downfield in shikimate, shikimate phosphate and EPSP. The signal at δ6.0 is an invariant ATP/ADP resonance

A purification of EPSP from the resulting mixture was achieved by anion exchange chromotography adapting the procedure used by Knowles and Sprinson in the preparation of EPSP from shikimate-3-phosphate.[19] The reaction mixture was first treated with apyrase (Sigma, grade VII), which degrades ATP and ADP to AMP and inorganic phosphate facilitating the isolation of uncontaminated EPSP[20] (ADP co-elutes with EPSP). The dibarium salts of (6*R*)-[6-^{2}H]EPSP and (6*S*)-[6-^{2}H]EPSP were prepared by precipitating with barium acetate,[19] in an overall isolated yield of 40% from **8**.[22] (6*R*)-[6-^{2}H]EPSP was used in kinetic studies of chorismate synthase, where a primary kinetic isotope effect was observed.[23]

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12. Using 2.5 equivalents of the amalgam minimised overreduction (less than 20%) to the propionic acid. The highest stereoselectivity obtained was 95%, obtained by the addition of the amalgam to a frozen solution of the bromoacrylate in D_2O, which was slowly warmed to room temp and then shaken vigorously for 25 minutes.
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14. The following axial coupling constants are seen in the ^1H NMR spectrum (250MHz, $CDCl_3$) of **7**; $J_{4,5}$=9 6 Hz, $J_{5,6(pro\ R)}$=11.6 Hz, $J_{1,6(pro\ R)}$=12 1 Hz
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16. For (6*R*)-[6-^{2}H]shikimic acid: δ_H (D_2O) 2.72 (1H, br, 6-H), 3.75 (1H, dd, $J_{4,5}$=8.4 Hz, $J_{4,3}$=4.3 Hz, 4-H), 4 02 (1H, dd, $J_{5,4}$=8.4 Hz, $J_{5,6}$=5.3 Hz, 5-H), 4.43 (1H, br. dd, $J_{3,4}$=4.3 Hz, $J_{3,2}$=4.2 Hz, 3-H), 6.70 (1H, dd, $J_{2,3}$=4 2 Hz, $J_{2,6}$=1.5 Hz, 2-H).
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20. Purification on a Dowex 1X-8 column (of dimensions 2 x 26 5 cm) eluting with a gradient of 0 to 0.6 M LiCl (in 0 01 M Tris-HCl, pH 9.0) at a flow rate of 0.6 ml min^{-1} and collecting 6 ml fractions typically gave the following elution profile (fraction number in brackets): shikimic acid (20-25, residual (+) enantiomer), inorganic phosphate (30-36), AMP (55-80), EPSP (98-110). All compounds were detected by the end absorption at 254 nm and their identity confirmed by ^1H NMR spectroscopy except inorganic phosphate which was detected colourimetrically (see reference **21**). No traces of shikimate-3-phosphate or phosphoenolpyruvate were detectable, however both elute no later than AMP
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22. For (6R)-[6-^{2}H]EPSP: δ_H (D_2O at pD=10.0) 2 89 (1H, br. d, $J_{6,5}$=5.2 Hz, 6-H), 4 01 (1H, dd, $J_{4,5}$=8.7 Hz, $J_{4,3}$=4 2 Hz, 4-H), 4 40 (1H, dd, $J_{5,4}$=8.7 Hz, $J_{5,6}$=5.5 Hz, 5-H), 4.69* (1H, d, $J_{8A,8B}$=2.5 Hz, 8A-H *trans* to carboxyl), 5 15* (1H, d, $J_{8B,8A}$=2.5 Hz, 8B-H *cis* to carboxyl), 6.48 (1H, d, $J_{2,3}$=4 2 Hz). 3-H signal masked by HOD
    * signals reduced in intensity due to deuterium incorporation from carrying out the EPSP synthase reaction in D_2O.
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